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PRINCIPAL INVESTIGATOR: Jonathan Chernoff, Ph.D.

CONTRACTING ORGANIZATION: Institute for Cancer Research
Philadelphia, PA 19111

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Jonathan Chernoff, Ph.D.

E-Mail: jonathan.chernoff@fccc.edu

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13. SUPPLEMENTARY NOTES

14. ABSTRACT
In this project, we are seeking to use induced pluripotent stem (iPS) cell technology as a potential therapy in NF1. In the first year, we have successfully produced iPS cells from fobroblasts from Nf1+/- mice and characterized the properties of these cells, which include growth in clusters, expression of stem cell markers, normal karyotype, and the ability to form teratomas in mice. We have also created a targeting "gene-repair" vector to replace the defective Nf1 allele in these iPS cells. Thus, we are on schedule for this project, having achieved our aims in the expected time frame.

15. SUBJECT TERMS
NF1, iPS, Schwann cells, gene targeting

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INTRODUCTION:

The goal of this project was to use induced pluripotent stem (iPS) cells and homologous recombination as the basis for therapy in neurofibromatosis 1 (NF1). Our hypothesis was that we could reprogram skin cells derived from NF1 heterozygous to become iPS cells, then repair the damaged NF1 allele, differentiate these iPS cells to hematopoietic precursors, and reintroduce such cells into irradiated *Krox20-Cre*; $Nf1^{flox/-}$ mice. The theory behind these experiments is that NF1-related tumors require not only $Nf1^{-/-}$ Schwann cells, but also $Nf1^{+/-}$ mast cells (1), and these could be replaced by the repaired iPS cells.

BODY: We set ourselves four specific tasks. These were:

Task 1. **Create iPS cells from $Nf1^{+/-}$ keratinocytes.** In this step, we will obtain keratinocytes from $Nf1^{+/-}$; Oct4-GFP mice, which should express GFP when converted to iPS cells.

1a. Cross $Nf1^{+/-}$ mice with Oct4-GFP mice to obtain $Nf1^{+/-}$; Oct4-GFP mice (months 1-4).
1b. Isolate and expand keratinocytes from 1-2 month old mice (months 4-6).
1c. Infect with four adenoviruses (encoding Oct4, Klf4, Sox2, and c-Myc, isolate and characterize iPS cells (months 6-12).

Task 2. **Repair the damaged *Nf1* allele.** We will use standard methods of homologous recombination to repair the damaged *Nf1* allele in $Nf1^{+/-}$ iPS cells.

2a. Construct *Nf1* (re)targeting vector (months 1-6).
2b. Transfect $Nf1^{+/-}$ iPS cells and identify targeted, $Nf1^{+/+}$ iPS cells. (months 12-15).

Task 3. **Convert the iPS cells to hematopoietic precursors.** To convert the undifferentiated iPS cells to a transplantable state, we will use HoxB4 to drive the cells towards a hematopoietic lineage.

3a. Infect iPS cells with GFP-HoxB4 retrovirus (month 15).
3b. Isolate embryoid bodies and grow cells on OP9 stromal feeder layer. (months 15-18).

Task 4. **Transplant NF1 mouse with iPS-derived $Nf1^{+/+}$ or $Nf1^{+/-}$ cells.** We will replace the bone marrow cells of Krox20-Cre; $Nf1^{flox/-}$ mice with marrow derived from repaired iPS cells and observe the animals for signs of disease.

4a. Irradiate Krox20-Cre; $Nf1^{flox/-}$ and transplant with hematopoietic precursors derived from iPS cells. (months 18).
4b. Observe mice for signs of disease. (months 18-24).

Progress

In the first year, we have achieved the first aim, and are well on our way to achieving aim two, as detailed below.

Task 1. **Create iPS cells from *Nf1*$^{+/-}$ keratinocytes.** We made several technical changes in the course of achieving this aim.

1a. Cross *Nf1*$^{+/-}$ mice with Oct4-GFP mice to obtain *Nf1*$^{+/-}$; Oct4-GFP mice (months 1-4). We omitted this step, as efficiencies of iPS production are now such we did not need to use an Oct4 reporter. We therefore did not carry out this cross.

1b. Isolate and expand keratinocytes from 1-2 month old mice (months 4-6) ___. We also omitted this step, as we found that we could efficiently reprogram mouse fibroblasts.

1c. Infect with four adenoviruses (encoding Oct4, Klf4, Sox2, and c-Myc, isolate and characterize iPS cells (months 6-12). We used retroviruses instead, as adenoviral transduction was not efficient in our hands. We used both the classic four factor (Oct4, Klf4, Sox2, c-Myc) and a three factor (Oct4, Klf4, Sox2) combinations to induce iPS formation. We found that the three-factor combination, which omits the potential oncogene c-Myc, gave robust, stem-cell appearing colonies (Fig. 1). Colony 3F-11, shown here, has a normal karyotype (not shown). This clone showed reactivation of endogenous stem cell genes, and formed embryoid bodies in vitro and teratomas *in vivo* (not shown).

Task 2. **Repair the damaged *Nf1* allele.** We are currently midway through this task, using standard methods of homologous recombination to repair the damaged *Nf1* allele in *Nf1*$^{+/-}$ iPS cells.

2a. Construct *Nf1* (re)targeting vector (months 1-6). We used recombineering to create a "repair" allele (Fig. 2). This allele uses hygromycin for selection, and G418 sensitivity for counter-selection. That is, correctly targeted alleles, in which the damaged, neo-containing NF1 allele are replaced with our floxed hygromycin cassette, will be identified as colonies of iPS cells that are hygromycin resistant but G418 sensitive.

2b. Transfect *Nf1*$^{+/-}$ iPS cells and identify targeted, *Nf1*$^{+/+}$ iPS cells. (months 12-15). This subtask is ongoing.

Task 3. **Convert the iPS cells to hematopoietic precursors.** We have not initiated this task yet, as it cannot be begun until task two is complete.

Task 4. **Transplant NF1 mouse with iPS-derived *Nf1*$^{+/+}$ or *Nf1*$^{+/-}$ cells.** We have not initiated this task yet, as it cannot be begun until task three is complete.

KEY RESEARCH ACCOMPLISHMENTS:

- Constructed pluripotent iPS cell lines from *Nf1*$^{+/-}$ cells.
- Completed retargeting construct to repair NF1 allele in these cells.

REPORTABLE OUTCOMES:

None to date.

CONCLUSION:

We are on schedule to complete our tasks. We made a number of changes along the way (e.g., use of fibroblasts in place of keratinocytes, use of retroviruses in place of adenoviruses, use of three reprogramming factors in place of four) to achieve successful production of pluripotent *Nf1*$^{+/-}$ iPS cells. In addition, we have successfully constructed a gene repair plasmid, which will be used to complete task 2.

The production of iPS cells from NF1 animals also allows us to differentiate these cells *in vitro* to Schwann cells. While not a part of this proposal, it is likely that such cells would be a useful resource for studying the role of NF1 in Schwann cell differentiation and in drug sensitivity studies.

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1. Yang, F.C., Ingram, D.A., Chen, S., Zhu, Y., Yuan, J., Li, X., Yang, X., Knowles, S., Horn, W., Li, Y., Zhang, S., Yang, Y., Vakili, S.T., Yu, M., Burns, D., Robertson, K., Hutchins, G., Parada, L.F., and Clapp, D.W., Nf1-dependent tumors require a microenvironment containing Nf1+/-- and c-kit-dependent bone marrow. *Cell*, **135**:437-448, 2008.

APPENDICES:

1) **Figure 1. Creation of iPS cells from *Nf1*$^{+/-}$ fibroblasts using three factors**
2) **Figure 2. NF1 gene repair vector**

Figure 1. Creation of iPS cells from *Nf1*$^{+/-}$ fibroblasts using three factors

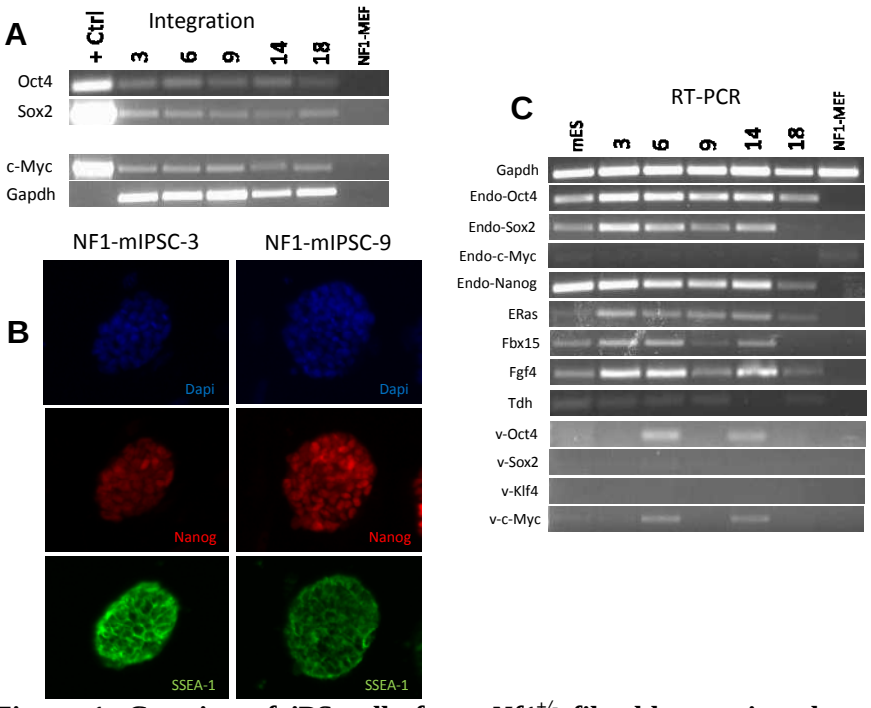

Figure 1. Creation of iPS cells from *Nf1*$^{+/-}$ fibroblasts using three factors. (**A**) RT-PCR analysis of expression of ste m cell markers. RNA was collected from cell lines 3, 6, 9, 14, and 18. (**B**) Staining of cells for expression of stem cell markers Nanog and SSEA-1 . (**C**) Expression of endogenous stem cell markers.

Figure 2. NF1 gene repair vector

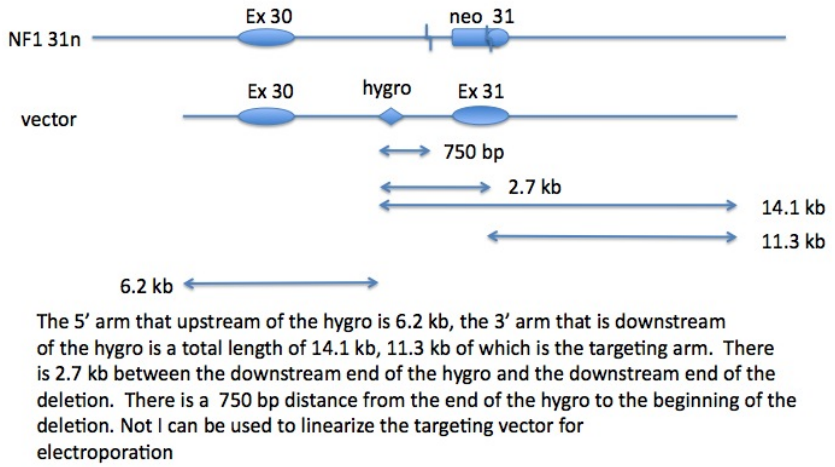

Figure 2. NF1 gene repair vector. The vector is designed to replace the disrupted NF1 allele in this mouse model, restoring exon 31.